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A METHOD FOR THE ROUTINE DETERMINATION OF GLYCOGEN IN OYSTERS

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It became necessary to develop a routine method for the determination of glycogen in oysters during a series of analyses of the Pacific coast species.

Pflüger's method for the digestion, precipitation, purification and hydrolysis was adopted as a basis of procedure and the possibility of using sodium hydroxide as a medium for digestion and some of the many recent modifications suggested by Sayhun and Alsberg (5), Good, Kramer, and Somogyi (3) and Cori and Cori (2) were investigated under the following headings.

In all cases the resulting glucose solutions were estimated by the Schaffer-Hartmann cuprous method (6) with the modifications of Marsh and Joselyn (4), and Schaffer and Somogyi (7).

1. Time of digestion in a 10 Normal solution of sodium hydroxide and potassium hydroxide, (Pflüger).

A number of fresh oysters were finely minced, thoroughly mixed, and four aliquot portions taken, two of which were digested in a 40 per cent solution of sodium hydroxide and two in a 56 per cent solution of potassium hydroxide (approximately 10 N.) at 100°C. One portion of each digest was heated for 3, and the others for 6 hours.

When cooled, all the digests were of an amber color with a curd of black soap floating on the surface. The sodium hydroxide digests were darker and the soaps

harder than those of the potassium hydroxide digests. The digests were diluted to twice their original volume, aliquots were withdrawn from each, and made 66 per cent with regard to ethyl alcohol, thoroughly mixed and the precipitates allowed to settle for 24 hours.

From the sodium hydroxide digests there was obtained a clear amber solution on top, and a black semi-liquid soap-like precipitate of impure glycogen at the bottom. The supernatant liquid was decanted through a # 2 Whatman filter with slight suction. There was no test for glycogen in the filtrate. The residue on reprecipitation yielded a yellowish flocculent precipitate of glycogen.

From the potassium hydroxide digest there resulted a layer of clear amber solution, and a gray-brown flocculent precipitate, which yielded pure white glycogen on reprecipitation.

- Table I -

The glycogen was then determined as glucose, and, as shown in Table I, the yields from both potassium and sodium hydroxide digests were no greater after 6 hours than after 3, consequently 3 hours' digestion was used in all subsequent analyses.

2. Concentration of ethyl alcohol required for complete precipitation of glycogen (3).

Aliquots of each of the sodium and potassium hydroxide digests were diluted to twice their original volume, made 40, 50 and 66 per cent with regard to ethyl alcohol, thoroughly mixed, allowed to settle for 24 hours, and the glycogen was then filtered off. In each case the concentration of alcohol in the filtrate was increased to determine the presence of unprecipitated glycogen. Identical results were obtained on the derivatives of both the sodium and the potassium

hydroxide digests.

There was additional precipitation of glycogen in the filtrate from the 40 per cent alcohol precipitation, when the concentration of alcohol was increased to 60 per cent but none when the 50 per cent filtrate was increased to 65 per cent or the 66 per cent was increased to 75 per cent.

- Table II -

The glycogen was purified by reprecipitation and determined as before. On the basis of these results, as shown in Table II, a concentration of 55-60 per cent ethyl alcohol was adopted for the glycogen precipitation.

3. Advantage of heating the alcohol solution of the digest in order to hasten the precipitation of glycogen (2).

The efficacy of heating the alkali solution after the addition of the alcohol as suggested by Cori and Cori (2) was tested by treating two aliquots from each digest, one from each as described above (Section 2), and the other by heating to boiling in a water bath. The glycogen when heated was precipitated as a gum-like substance and adhered to the sides of the vessel. The supernatant liquids, when decanted twice through a Whatman # 2 filter with slight suction, were each perfectly clear and gave no test for glycogen. All samples were reprecipitated as before, and the glycogen determined.

- Table III -

As shown in Table III, both methods gave identical results, consequently the heating procedure was adopted in further tests in order to hasten precipitation.

4. Concentration of hydrochloric acid and the time of digestion at 100°C. necessary for the complete hydrolysis of the glycogen (5).

Sayhun and Alsberg (5) show that complete hydrolysis is accomplished by heating the glycogen solution at 100°C. for 3 hours with 2.2 per cent (0.6 N) and in 2 hours with 3.65 per cent (N) hydrochloric acid.

Concentrations of 1 per cent (0.27 N), 3 per cent (0.83 N), and 5 per cent (1.38 N) hydrochloric acid were used for the hydrolysis of the glycogen solutions obtained from each digest, and the times of digestion were chosen as 1, 2, and 3 hours at 100°C. The results are shown in Table IV.

- Table IV - .

It is assumed that the values of glycogen expressed as glucose (26.1 per cent), represents the nearest estimation to the true analysis. For the temperature of digestion, namely 100°C., it is shown that the largest yield of glucose is obtained from the potassium hydroxide digest with 3-5 per cent hydrochloric acid in 3 hours.

A subsequent test was made with 3 per cent hydrochloric acid for 5 hours but there was no further hydrolysis, and there appeared to be a small but definite loss of glucose.

5. Tendency toward adsorption of the glycogen by the soaps formed during the digestion, (1), (8), (9).

It is shown in Tables I, II, III and IV that the yield of glucose from the sodium hydroxide digestion is much less than that from the potassium hydroxide digest. This discrepancy is possibly due to the adsorption of glycogen on the sodium soap formed during the digestion and to determine the extent of such adsorption, if any, a separate examination of the soap and the supernatant liquid was made in both digests. The results are shown in Table V.

- Table V -

This indicates that the glycogen makes a definite selection of the supernatant liquid in the sodium hydroxide digest, or that glycogen is adsorbed on the soap and does not yield to hydrolysis in this state.

Schöndorf (8) has shown that glycogen is readily adsorbed on the hydroxides of the heavy metals which occur in oysters, and states that determination by Pflüger's method may be as much as 50 per cent too low from this cause. Przylecki and Mazmiu (9) show that it is adsorbed on fats and lecithin, from which it may be reasoned that the colloidal glycogen may be readily adsorbed by the soap in the digest. Bancroft and Fry (1) have demonstrated that the extent of hydrolysis depends on the amount of unadsorbed glycogen and is independent of a large excess of either acid or glycogen, and the results they present are very similar to those shown in Table V. It is evident that the error would be much greater when dealing with small quantities than with large.

- Using potassium hydroxide there is apparently no difference in the concentration of glycogen in any part of the digest, hence this reagent was adopted, and the aliquots for routine analyses were drawn from the supernatant liquid merely to simplify the purification procedure.

6. Necessity of filtering the glucose solution before determination.

When the glucose solution obtained from the acid hydrolysis of the glycogen is neutralized, a dark flocculent precipitate is formed. The glucose is a neutralized solution derived from the potassium hydroxide digest was determined in two aliquots, only one of which was filtered.

The filtered sample yielded 20.7 per cent and the unfiltered sample 19.9 per cent glucose, demonstrating that the precipitate interferes with the glucose

estimation.

7. Use of glucose for the standardization of the sodium thiosulphate solution in the Schaffer-Hartmann cuprous procedure (6).

In the determinations of glucose on the macro scale by the Schaffer-Hartmann cuprous method (6), copper is used for the standardization of the sodium thiosulphate solution. A comparison was made, substituting glucose for copper.

The amount of glucose in a sample of "Difco" standardized anhydrous dextrose was determined in the prescribed manner. Corrections were made in the weight taken for the stated impurities, and the normality of the sodium thiosulphate solution was calculated.

This normality was 0.1526, and referred to pure copper was 0.1528, from which it is evident that either substance is suitable for this standardization.

Procedure Adopted

On the basis of the foregoing investigation, the following procedure was adopted for the routine determination of glycogen in oysters.

Preparation of the sample.

Shuck and drain a number of oysters sufficient to give a suitable quantity of the moist meat. Mince finely, including any liquor that is ground out of the oysters, mix and sample the resultant mass.

Digestion.

Weigh 20 grams of the wet sample into a 200-ml. Berzelius beaker, add 40-50 ml. of hot 56-60 per cent potassium hydroxide, provide a cover and stirring rod, and digest with frequent stirring in a boiling water bath for 3 hours. (Note: No reflux is required as the concentrated alkali will absorb enough

water from the steam of the water bath to keep the volume approximately constant.) Cool, and dilute the digest to 100 milliliters.

Precipitation

Withdraw 50 ml. of the amber liquid, neglecting the black scap, into a 200 ml. Berzelius beaker, add 80 ml. of 95 per cent ethyl alcohol and stir well. Heat in a water bath (about 80°C.) until the alcohol in the mixture begins to boil. The precipitated glycogen adheres to the sides of the beaker. Decant the supernatant liquid through a #2 Whatman filter under slight suction, repeating if necessary until the filtrate is bright and clear.

Purification.

Redissolve the raw glycogen in 50 ml. of boiling water and reprecipitate. This second precipitate should be very white in an almost colorless liquid. Heat as before in the water bath, and decant the supernatant liquid through the same filter. Wash the paper into the beaker with boiling water and redissolve the pure glycogen, making the solution up to 100 ml. (approximately). The glycogen solution, when cool, should be opalescent and have a very light buff color.

Hydrolysis.

Add 8.5 to 11 ml. concentrated hydrochloric acid, making the solution 3-4 per cent with respect to the acid, and digest in a boiling water bath for 3 hours. Cool, add a drop of 2 per cent phenolthalein solution to the glucose solution, and add concentrated potassium hydroxide until a pink color is just perceptible. Filter with slight suction, wash with boiling water, and make the filtrate up to 250 ml. The glucose solution should be bright, clear, and have a very light buff color (or pink, due to the phenolthalein).

Determination.

The glucose may now be determined by any of the standard methods. If the Schaffer-Hartmann procedure (6) is adopted, the sodium thiosulphate solution may be standardized with copper, or pure dextrose as described.

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TABLE I

Digestion Period (hours)	Sodium Hydroxide Digest (per cent of glycogen as glucose)	Potassium Hydroxide Digest (per cent of glycogen as glucose)
3	15.5	19.8
6	15.5	19.8

TABLE II

Concentration of Ethyl Alcohol (per cent)	Sodium Hydroxide Digest (per cent of glycogen as glucose)	Potassium Hydroxide Digest (per cent of glycogen as glucose)
40	5.5	11.2
50	15.5	19.8
66	15.5	19.8

TABLE III

55-60 Per Cent Alcohol Precipitation (state)	Sodium Hydroxide Digest (per cent of glycogen as glucose)	Potassium Hydroxide Digest (per cent of glycogen as glucose)
Cold	15.5	19.8
Hot	15.5	19.8

TABLE IV

Concentration of Hydrochloric Acid (per cent)	Time of Hydrolysis (hours)	Sodium Hydroxide Digest (per cent of glycogen as glucose)	Potassium Hydroxide Digest (per cent of glycogen as glucose)
1	1	7.16	16.9
3	1	13.5	19.9
5	1	19.1	24.5
1	2	11.9	23.0
3	2	13.5	24.5
5	2	11.1	24.5
1	3	11.9	23.7
3	3	15.1	26.1
5	3	15.9	26.1
3	5	--	26.0

TABLE V

	Sodium Hydroxide Digest (per cent of glycogen as glucose)	Potassium Hydroxide Digest (per cent of glycogen as glucose)
Supernatant liquid	15.3	20.1
Black soap	6.8	20.1